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SHORT COMMUNICATION

Two-dimensional zymography in detection of proteolytic enzymes in wheat leaves

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Abstract Proteolytic enzymes in wheat leaves were studied using zymographic detection of enzyme activities on one-way (1D) SDS-polyacrylamide gels and two-dimensional (2D) ones, on which protein samples were isoelectrofocused prior to PAGE separation. Gelatin of concentration 0.1 %, copolymerized into SDS-PAGE gels, digested by active proteinases enabled detection of those enzymes. On 1D gels, seven bands were seen and assigned to particular families through the use of specific inhibitors. Metalloproteinases inhibited by 20 mM EDTA were detected as 150 kDa band; aspartic proteinases were assigned to 115–118 kDa double band by using 25 mM pepstatin; 10 mM phenylmethylsulfonyl fluoride used for detection of serine proteinases pointed to band of 70 kDa and finally due to 10 μM E-64 inhibitor, cysteine proteinases of 37 and 40 kDa were detected. On 2D gels, additional separation according to protein isoelectric points enabled detection of proteinase isoforms. In the range of 4.5–6 p*I*, six metalloproteinases as well as ten aspartic proteinases were visible, ten serine- isoforms of p*I* 4.5–6.8 and four cysteine proteinases of 4.5–5.0 p*I* were found. Presented results were detected as reproducible results observed at least in four independent biological replications.

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Introduction

Zymography is a widely accepted analytical technique used to detect enzymes in tissue extracts. It is based on visualization of areas where the specific substrate is digested by the enzyme of interest. It can be used to perceive the total enzymatic activity or to see activities of enzyme isoforms following the protein electrophoretical separation on polyacrylamide gels (Vandooren et al. 2013).

The most popular systems, arranged in a hierarchy of Google search results, have been elaborated for detection of: phosphatases (Robinson and Glew 1980), reactive oxygen species scavenging enzymes: catalases, superoxide dismutases, peroxidases (Shukla et al. 2009; Srivalli and Khanna-Chopra 2001), amylases (Steup and Gerbling 1983) and proteinases (Lantz and Ciborowski 1994). Furthermore, the system has been successfully used for detection of glycohydrolases: glucanases and glucosidases, xylanases, mannases, hemicellulases, cellulases and ligninases (Joo et al. 2009; Royer and Nakas 1990; Sakamoto and Toyohara 2009) as well as esterases, lipases (Kwon et al. 2011) and nucleases (Cazenave and Toulme 2001). A crucial role in detection of enzymes activities using zymography play inhibitors. For detection of proteolytic enzymes, for example, the comparison of gel patterns from the substrate digestion without the use of inhibitor to gels on which digestion was carried out in the presence of specific inhibitor allows the identification of a band that corresponds to the enzyme of interest.

Most commonly, for proteinase activity detection after denaturing but nonreducing electrophoresis, protein

renaturation is followed by substrate digestion (Zhang and Jones 1995). All of these are carried out in the gel soaked in a buffer of pH appropriate to the pH optimum of studied enzyme. A modification of this method is the detection of proteinase inhibitors in the gel. While such a gel is incubated in a buffer containing the enzyme of interest nearly the whole substrate incorporated into the gel is digested beside the places where enzyme inhibitors are present. Such a method is commonly used to detect metalloproteinase inhibitors and is named reverse zymography (Hawkes et al. 2010). Usage of fluorescence-labeled matrix protein improved this technique by enabling the in situ inhibitor visualization, in histological sections (George and Johnson 2010).

Even though the progress in proteomic methods is rapid, zymography still has many advantages. This method is relatively cheap and fast, moreover, it enables immediate location of studied enzymes. However, in some cases, separation of protein from the gel is less accurate. When high intensity patterns are detected some isoforms of the protein could not be visible. Proteins could migrate slower through the gel containing substrate, influencing on molecular weight overestimation. On the other hand, conditions of extraction and electrophoresis may influence the reduction of enzyme activities leading to underestimation of their amount (Pan et al. 2011). In this short communication, we report the advantage of using two-dimensional (2D) zymography in combination with specific proteinase inhibitors to reveal the pattern of major leaf proteases in wheat. We demonstrate that zymography is still the technique of choice in protease studies and permits very good separation of distinct proteinase families.

Materials and methods

Plant material

The experiments were carried out on 10-day-old seedlings of spring wheat (*Triticum aestivum* L.) $Ethos_{cult}$, grown as described previously (Grudkowska and Zagdanska 2010). In brief: fourfold replica of 25 germinated grains were placed side by side between double filter paper strips, rolled up and grown in plastic boxes in a climatic chamber at conditions of day/night temperature 18/14 °C, photoperiod 16/8 h, photosynthetic photon flux density: 260 μmol m^{-2} s^{-1} and 70–80 % air humidity. Seedlings were fertilized with Knopp solution supplemented with Hoagland's micronutrients.

Protein extraction

Prior to extraction, 300 mg of leaves were ground in liquid nitrogen followed by grinding in 1 ml of extraction buffers: (1) denaturing non-reducing lysis buffer containing primarily 6 M urea, 2 M thiourea, 0.05 % CHAPS 0.5 % and carrier ampholytes (BioRad, Bio-Lytes p*I* 3–10), according to the BioRad Manual and in subsequent experiments in the buffer with amount of urea decreased to 4 M to improve zymography results, or (2) non-denaturing non-reducing 50 mM sodium acetate buffer of pH 5 containing 1 mM EDTA and 2 mM cysteine, common for vacuolar protein extraction prior to one-dimensional (1D) electrophoresis (Zhang and Jones 1995). Extractions were followed by 15 min centrifugation at 4 °C and 15,000*g*. Soluble proteins were precipitated from the supernatant with 100 % ice-cooled acetone, separated by centrifugation for 10 min at 15,000*g*, 4 °C, washed twice in 80 % acetone, air-dried and stored at −80 °C until used.

IEF and SDS-PAGE-electrophoresis

For isoelectric focusing (IEF), two types of buffer were used alternatively: (1) denaturing non-reducing: 4 M urea, 2 M thiourea, 0.5 % CHAPS, 0.001 % bromophenol blue, 0.25 % carrier ampholytes (BioRad, Bio-Lytes p*I* 3–10), or (2) non-denaturing non-reducing IEF sample buffer containing 1 % NP-40, 5 % glycerol, 0.001 % bromophenol blue, 0.25 % carrier ampholytes (BioRad, Bio-Lytes p*I* 3–10). IPG-Dalt strips (BioRad) in the pH range 4–7 were rehydrated overnight under passive conditions. Each of the operation was carried in one of the two rehydratation buffers. 60 μg of protein (determined spectrophotometrically at 595 nm by Bradford method with BSA as a standard) was carried out on the Immobiline Dry Strip. For PAGE electrophoresis, protein was dissolved in distilled water and mixed 1:1 with SLB.

Isoelectric focusing (IEF) was ran using a Biorad PROTEAN IEF focusing chamber at a constant temperature of 20 °C and at 50 μA/strip for 20 min at 250 V followed by a 2 h linear ramping to 4,000 V and final focus until the value of 10,000 Vh was reached. Strips were frozen and the next day were equilibrated in 50 mM Tris-HCl incubation buffer, pH 6.8, containing 4 M urea, 30 % glycerol, 2 % SDS for 15 min. After equilibration, strips were sealed on the top of 10 % SDS-PAGE gels using 0.5 % agarose in 0.1 M Tris-HCl pH 6.8 containing 0.001 % bromophenol blue. SDS-PAGE was run on 10 % acrylamide gels with 0.1 % gelatin copolymerized prior to zymography, in 50 mM Tris-Cl buffer pH 6.8, at constant amperage of 20 mA/gel (mini-gel 80 mm × 60 mm × 0.75 mm) until the blue dye front reached the bottom of the gel. Molecular weight standard PageRuler ™ (Thermo SCIENTIFIC) was used for electrophoresis in 2nd dimension.

Zymography

Reactivation of protein after electrophoretical separation was achieved by the double incubation of gels in 2.5 %

Tween 20 for 30 min. Afterward gels were briefly rinsed three times with distilled water followed by 16 h incubation in 50 mM acetic buffer pH 5.0 containing 2 mM cysteine prior to digestion of copolymerized gelatin by proteolytic enzymes. Incubations were carried out with gentle shaking at 25 °C. Gels were then rinsed with water as before and stained for 3 h in staining solution (0.5 % amido black, 30 % methanol, 10 % acetic acid). Finally, gels were destained in solution of 30 % methanol and 10 % acetic acid until clear bands were visible on a dark blue background. To detect proteinase specificity enzyme, inhibitors were used: 20 mM EDTA for metalloproteinases (EC 3.4.24), 25 mM pepstatin for aspartic proteinases (EC 3.4.23), 10 mM phenylmethylsulfonyl fluoride (PMSF) for serine proteinases (EC 3.4.21), and 10 μm E-64 for inhibition of cysteine proteinases (EC 3.4.22) (Zhang and Jones 1995). Protein extracts were incubated for 2 h in buffer of pH 5, containing appropriate inhibitor prior to electrophoresis, as well as after electrophoreses, the gelatin digestion was carried out in the presence of those inhibitors. The presented results were detected as reproducible results observed at least in four independent biological replications.

Results and discussion

Since extraction and zymographic detection of proteinases in recommended conditions (Rossano et al. 2011; Wilkesman and Schroder 2007) in denaturing and non-reducing the buffer did not yield expected results showing only four spots of MW about 70 kDa and differing in p*I* (Fig. 1a), protein extraction was performed in non-denaturing, non-reducing environments. In such conditions, seven groups of proteinases differing in MW as well as p*I* were detected.

To specify the proteinase families (as named in MEROPS database http://merops.sanger.ac.uk,) or sub-subclasses (according to International Union of Biochemistry and Molecular Biology: IUBMB/1992 www.chem.qmul.ac.uk/iubmb/enzyme/) on 2D zymograms, it was decided to detect the proteinases by zymographic assays performed on 1D gels (Fig. 2). Protein separation by 2D electrophoresis requires two consecutive, discrete steps: IEF followed by SDS-PAGE. The enzyme activity bands as well as their decay in the presence of specific inhibitors are easier to be detected on 1D SDS-PAGE gels than the spots resulted from 2D separation. Since SDS-PAGE in both 1D and 2D separations were run in the same conditions; proteinase family identification was performed on 1D zymograms (Fig. 2) and extrapolated by the 2D technique (Fig. 1b). Zymographic detection of proteinases on the SDS-PAGE gel revealed 7 bands in control leaf extract non-treated by specific inhibitors.

The use of 20 mM EDTA, inhibitor of metalloproteinases (EC 3.4.24), suppressed single, 150 kDa band (EP1). Metalloproteinases are ATP-dependent enzymes, having the divalent cation of zinc, rarely cobalt, in the active center with chaperone-like activity in the ATPase domain. These enzymes are well characterized in mammals as membrane proteins, crucial for housekeeping as well as for recovery from pathological processes (Mariano and Funk 2012). Recently, they have been identified in oligomeric complexes of plant mitochondrial and chloroplastic membranes: in mitochondria, as part of cytochrome *bc1* and in chloroplasts as part of heterocomplex degrading protein *D1*, the core protein of photosystem II (PS II). In *Arabidopsis*, 12 genes encoding proteins homologous to *Escherichia coli* FtsH metalloproteins were identified (Janska et al. 2013; Lucinski and Jackowski 2013; Piechota et al. 2010; Wagner et al. 2012). In broccoli florets, metalloproteinases of MW 69 kDa were detected by 2D zymography (Rossano et al. 2011). From soybean leaves, *Slti114* gene encoding metalloproteinase of 44 kDa and predicted 5.5 p*I* was isolated but zymographic detection of protein product was unsuccessful (Cho et al. 2009).

The use of 25 mM pepstatin A, the naturally occurring hexapeptide, highly specific and competitive suppressed aspartic proteinase (EC 3.4.23) double band (EP2-EP3) of molecular weight 115–118 kDa. Aspartic proteinases are named based on IUBMB/1992 classification (www.chem.qmul.ac.uk/iubmb/enzyme/) which refers to mechanisms of catalytic reaction. In the MEROPS database, peptidases are grouped into families according to a hierarchical, structure-based classification referring to statistically significant similarities in amino acid sequence further grouped by homology into clans (Rawlings et al. 2012). Aspartic proteinases belong to families C14A (caspases) and C14B (metacaspases) of CD Clan (Misas-Villamil et al. 2013) which makes them the most studied proteinases due to their participation in processes of apoptosis and programmed cell death (PCD) (Chichkova et al. 2012; Marek 2013; Ouyang et al. 2012; Shrestha and Megeney 2012; Tsiatsiani et al. 2011; Wang and Bayles 2013). In wheat grain, aspartic proteinases were detected by Tamura et al. (2007). Authors have mentioned two isoforms of 35 and 27 kDa isolated by affinity chromatography in denaturing conditions. Type II metacaspase from wheat leaves (TaeMCAII) was modeled in silico, cloned and characterized (Piszczek et al. 2011; Piszczek et al. 2012). Studies of *Arabidopsis thaliana* dwarf mutant, impaired in carbohydrate metabolism, enabled characterization of NANA, chloroplast-located, aspartic proteinase (Paparelli et al. 2012).

Phenylmethylsulfonyl fluoride (PMSF) is an inhibitor of serine proteinases (EC 3.4.21). Although usually it does not inhibit all serine proteinase activity, due to a short half-life in water solutions, it is commonly used in detection of

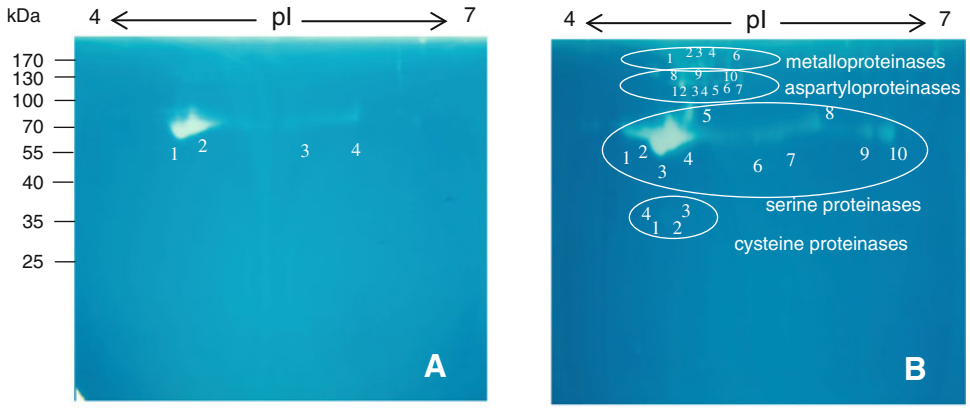

Fig. 1 Zymographic detection of wheat seedling proteinases separated by 2D electrophoresis in conditions: **a** denaturing and non-reducing (4 M urea, 2 M thiourea, 0.5 % CHAPS, 0.001 % bromophenol blue, 0.25 % carrier ampholytes) and **b** non-denaturing and non-reducing (1 % NP-40, 5 % glycerol, 0.001 % bromophenol blue, 0.25 % carrier ampholytes). Spots of detected proteinases are numbered

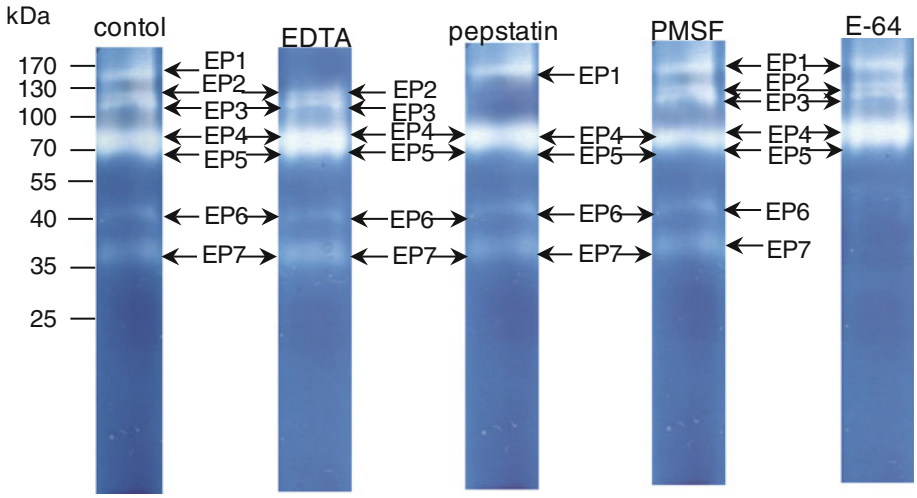

Fig. 2 Zymographic identification of proteases families wheat seedlings on 1D gels using specific inhibitors: 20 mM EDTA for inhibition of metalloproteinases: 25 mM pepstatin for aspartic proteinases, 10 mM PMSF for serine proteinases and 10 μM E-64 for inhibition of cysteine proteinases. Experiment was carried out in non-denaturing non-reducing conditions. Sample non-treated by any inhibitor was a control. Bands of proteinase activities are marked by *arrowheads*

those proteinases. The molecular weights of double EP4 and EP5 bands in which intensity was decreased upon action of this inhibitor is about 70 kDa. Rossano et al. (2011) detected 56 kDa bands of serine proteinase in senescence broccoli florets. In spinach chloroplasts, two subunits of alkaline serine proteinase characterized by molecular weights 63 and 32 kDa and maximal activity in 50 °C at pH 8.5 were purified using the HIC–HPLC system. When separated on native PAGE, only a single band of 95 kDa was visible (Srivastava et al. 2009). Acting in parallel to the carboxy-terminal processing proteinase CtpA and the ATP-dependent thylakoid-bound metalloproteinase FtsH, the serine-type proteinase DegP is critical for the biogenesis and maintenance of PSII (Chi et al. 2012).

The irreversible inhibitor of cysteine proteinases, E-64, that binds covalently to the enzyme reactive center was used to detect their bands on a 1D zymogram. Disappearance of EP6 and EP7 bands of MW about 37 and 40 kDa was indicated on cysteine proteinases enzymes (Fig. 2). From germinating barley grains, cysteine proteinase of MW 31 kDa was purified and 4.4 p*I* was detected by 2D zymography (Zhang and Jones 1996). In broccoli florets, cysteine proteinase bands of 48 and 44 kDa and p*I* 4.4 and 4.2 were detected. By 2D zymography, 11 cysteine proteinases were revealed using 7 M instead of 4 M urea in strip rehydratation solution. Those conditions conversely influenced the irreversible denaturation of serine proteinases and metalloproteinases, which disappeared from zymograms (Rossano et al. 2011). In kiwi fruits, 33.8, 32.2, and 31.4 kDa bands of cysteine proteinases at p*I* 3.92 (Larocca et al. 2010) as well as a wide band of actinitin of MW about 30 kDa (Afshar-Mohammadian et al. 2011) were found. Maciel et al. (2011) detected a 29 kDa proteinase having acidic optimal pH and the 38 kDa proteinase with neutral optimum activity in wounded and methyl jasmonate-treated leaves of *Ricinus communis* (L.). Induction of those isoforms by jasmonate suggested a possible role of cysteine proteases in leaf senescence. Intensive experiments and in silico studies by van der Hoorn group resulted in classification of 723 plant papain-like cysteine proteinases available in MEROPS DB, into

nine subfamilies (Richau et al. 2012). Those enzymes are highly resistant, which allows them to act in proteolytically harsh environments of apoplasts, vacuoles, or lysosomes during cell aging or apoptosis. Most known proteases: SAG12 (Grbić 2003) and RD21 (Hara-Nishimura et al. 1995) have been structurally characterized as having double cysteine or two subfamily-specific disulfides in the catalytic site, respectively (Richau et al. 2012; Misas-Villamil et al. 2013).

Side-by-side comparison of 1D and 2D gels made it possible to identify various proteinase families on 2D gels. Metalloproteinase isoforms visible on the top of the gel in the range 4.5–6.0 p*I* were counted as seven spots. Aspartic proteinases of 115–118 kDa showed 10 spots in the same range of p*I*. Ten serine proteinase spots were assigned to molecular weight of 80 kDa (two spots) and 70 kDa (eight spots). Also cysteine proteinases were shown to be presented in two separate subfamilies of 37 and 40 kDa.

There are various techniques available for proteinases identification and characterization, among which zymography has been one of leading due to feasibility of active isoform isolation from the gel (Vandooren et al. 2013). Zymography, especially when run on 1D gels, is a relatively inexpensive technique, which requires only specific inhibitors of costly reagents. Both variants 1D and 2D, if used in combination with other techniques, help in understanding the functions of the enzyme of interest in various physiological processes (Vandooren et al. 2013). In studies of wheat resistance to abiotic stresses inducing tissue dehydration, zymography can be used for detection of proteolytic enzymes (Grudkowska and Zagdanska 2004). While in response to abiotic stress, there is a need to degrade of redundant, misfolded or damaged protein; proteinases play the main role conducting proteolysis, a chemical reaction that breaks peptide bonds releasing peptides and amino acids. Under environmental conditions inducing tissue dehydration (mainly drought, cold, frost) proteome rebuilding is an important mechanism of plant restoration and tissues homeostasis maintenance. Use of zymography allows the identification of enzyme families on 1D zymograms and number of proteinase subfamilies and clans using 2D gels (Rawlings et al. 2012).

Author contribution MG and KR declare equal participation in the implementation of the work. PL, the MSc student, was guided in carrying out part of experiments.

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